PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark Office, PCT
2011 South Clark Place Room
CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 02 November 2000 (02.11.00)	
International application No. PCT/EP00/01878	**Applicant's or agent's file reference** 10631
International filing date (day/month/year) 06 March 2000 (06.03.00)	**Priority date** (day/month/year) 09 March 1999 (09.03.99)
Applicant FLOHÉ, Leopold et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

09 October 2000 (09.10.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer

Juan Cruz

Telephone No.: (41-22) 338.83.38

9/914584

PATENT COOPERATION TREATY

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
10631		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP00/01878	06/03/2000	09/03/1999
International Patent Classification (IPC) or national classification and IPC C12Q1/28		
Applicant FLOHE, Leopold et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 3 sheets.

3. This report contains indications relating to the following items:

I	☒	Basis of the report
II	☐	Priority
III	☒	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
IV	☐	Lack of unity of invention
V	☒	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
VI	☐	Certain documents cited
VII	☒	Certain defects in the international application
VIII	☒	Certain observations on the international application

Date of submission of the demand	Date of completion of this report
09/10/2000	08.06.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Weijland, A Telephone No. +49 89 2399 7490

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP00/01878

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-22	as originally filed

Claims, No.:

1-7	as received on	14/05/2001	with letter of	09/05/2001

Drawings, sheets:

1/4-4/4	as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   - ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   - ☐ the language of publication of the international application (under Rule 48.3(b)).
   - ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   - ☐ contained in the international application in written form.
   - ☐ filed together with the international application in computer readable form.
   - ☐ furnished subsequently to this Authority in written form.
   - ☐ furnished subsequently to this Authority in computer readable form.
   - ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   - ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   - ☐ the description, pages:
   - ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 5-7.

because:

☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (*specify*):

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☒ no international search report has been established for the said claims Nos. 5-7. (See separate sheet)

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

☐ the written form has not been furnished or does not comply with the standard.

☐ the computer readable form has not been furnished or does not comply with the standard.

V. R asoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N) Yes: Claims 1-4

	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-4
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-4
	No:	Claims	

2. Citations and explanations
see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

The following documents (D) are referred to in this report:

D1: MAIORINO M. ET AL.: 'Testosterone mediates expression of the selenoprotein PHGPx by induction of spermatogenesis and not by direct transcriptional gene activation' FASEB J., vol. 12, 1998, pages 1359-1370, XP002141807

D2: WO 96 13225 A (BETH ISRAEL HOSPITAL ASSOCIATION) 9 May 1996 (1996-05-09)

D3: ROVERI A. ET AL.: 'Enzymatic and immunological measurements of soluble and membrane bound PHGPx' METHODS ENZYMOL., vol. 233, 1994, pages 202-212, XP000921475 cited in the application

D4: MAIORINO M. ET AL.: 'Phospholipid hydroperoxide glutathione peroxidase' METHODS ENZYMOL., vol. 186, 1990, pages 448-457, XP000921458

SECTION III

1. No International Search Report has been established for present claims 5-7, therefore they will be not subject of an International preliminary examination (Rule 66.1(e) PCT).

SECTION V

2. The subject matter of claims 1-4 is novel (Article 33(2) PCT).

   D1 to D4 are cited in the International search report as documents of particular relevance to the present set of claims. However, claim 1 differs from D1 to D4 in that it describes a method for screening inhibitors of hydroperoxide gluthathione peroxidase that as part of a pharmaceutical acceptable inhibitor, reversibly suppresses male fertility. Claim 1 is therefore not disclosed in the prior art documents.

3. The subject matter of claims 1-4 would appear to involve an inventive step (Article 33(3) PCT).

D1 is considered to be the closest prior art. D1 (abstract; page 1360, left column, first paragraph; page 1368, left column, third paragraph, right column, second paragraph) describes that selenium deficiency is shown to be associated with male infertility and the selenoprotein PHPGPx has shown to increase in rat testis after puberty and to depend on gonadotropin stimulation in hypophysectomized rats. There is a striking burst of PHGPx expression at the transition of round to elongated spermatids that ceases abruptly in elongated spermatoids, what suggests a role of this selenoprotein in sperm maturation, however one awaits still a final proof. The idea is strengthened that its peculiar expression in testis is somehow related to spermatid differentiation, that might explain the male infertility observed in selenium deficieny, a complex process in which other selenoproteins might complement PHGPx in the testicular maturation process. Claim 1 differs from D1 in that it describes a method for screening for inhibitors of PHGPx comprising steps a) to c) to search inhibitors specifically blocking PHGPx to suppress male fertility.

The technical problem to be solved would appear to reside in finding substances which reversibly suppress male fertility.

The skilled person, equipped with the knowledge of D1, would not be motivated to arrive at the subject matter of claim 1, i.e. a method for screening inhibitors of PHGPx, despite its potential role in the maturation process of spermatozoa has been described in D1 (see above) and enzymatic assays and inhibitors are known from D1 (page 1360, right column, third and fourth paragraph) and D3 (page 204, last paragraph, page 205 and page 206, first paragraph), since it has not been suggested in the prior art documents , that in late stages of sperm maturation PHGPx is oxidatively cross-linked to become a structural element indispensible for sperm function (see page 2, first paragraph) and any shortage of PHGPx during sperm maturation, be due to selenium deficiency or inhibition of activity should therefore result in disturbed sperm midpiece architecture and in consequence in loss of fertilization potential of sperm. The same applies to claims 2-4, dependent on claim 1.

SECTION VII

4. Contrary to the requirements of Rule 5.1(a)(ii) PCT, the relevant background art disclosed in D1 is not mentioned in the description, nor is this document identified therein.

5. The amount of bands in Figure 1 c does not correlate with the passage on page 4 (line 9) of the description,which states that four bands are present.

SECTION VIII

6. The vague and imprecise statement in the description "and all changes which come within the meaning and range of equivalency of the claims..." on page 3 (lines 25-28) of the description implies that the subject-matter for which protection is sought may be different to that defined by the claims, thereby resulting in lack of clarity (Article 6 PCT) when used to interpret them (see also the PCT Guidelines, PCT/GL/3 III, 4.3a).

April 18, 2001/bu

International Patent Application PCT/EP 00/01878
Leopold Flohé et al.

New Claims

1. Method for screening for pharmaceutically acceptable PHGPx inhibitors (inhibitors of phospholipid hydroperoxide glutathione peroxidase), which, by specifically blocking PHGPx, reversibly suppress male fertility, comprising the steps of

(a) determining the enzymatic activity of said PHGPx, which is derived from human tissue or human cells, in the absence and presence, respectively, of at least one potential inhibitor,

(b) selecting at least one inhibitor which specifically blocks PHGPx activity and subjecting said inhibitor(s) to a screening for pharmaceutical acceptance, and

(c) selecting a pharmaceutically acceptable inhibitor.

Claims

1. Method for screening for inhibitors of phospholipid hydroperoxide glutathione peroxidase (PHGPx) derived from human tissue or human cells comprising the steps of
   a) determining the enzymatic activity of said PHGPx in the absence and presence, respectively, of at least one potential inhibitor,
   b) selecting at least one inhibitor which specifically blocks PHGPx activity and subjecting said inhibitor(s) to a screening for pharmaceutical acceptance and
   c) selecting a pharmaceutically acceptable inhibitor which, by specifically blocking PHGPx, reversibly suppresses male fertility.

2. Method of claim 1, wherein the tissue or cells are from life stock or any related mammalian species.

3. Method of claim 1, wherein PHGPx is produced by genetic engineering.

4. Method of claim 1, 2 or 3, wherein the potential inhibitors have been tailored by computer designing and/or produced by a chemical process of production.

5. A pharmaceutically acceptable inhibitor of PHGPx from human tissue obtainable by the method according to claim 1, 2, 3 or 4 and useful for male fertility control.

6. Pharmaceutical composition comprising at least one inhibitor of PHGPx from human tissue according to claim 5

and at least one pharmaceutically acceptable carrier and/or diluent or no such carrier/diluent.

7. Use of an inhibitor of PHGPx according to claim 5 or of a pharmaceutical composition comprising said inhibitor of PHGPx according to claim 6 in a method for reversibly blocking male fertility.

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

Boeters,Hans
BOETERS & BAUER
Bereiteranger 15
D-81541 München
ALLEMAGNE

EINGEGANGEN
11. Juni 2001
BOETERS & BAUER
Patentanwälte

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	08.06.2001
Applicant's or agent's file reference 10631	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP00/01878	06/03/2000	09/03/1999

Applicant
FLOHE, Leopold et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Neumann, M Tel.+49 89 2399-7351

Form PCT/IPEA/416 (July 1992)

EL 905051675 US

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
10631		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/EP 00/ 01878	06/03/2000	09/03/1999
Applicant		
FLOHE, Leopold		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 5 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [X] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. 1

   [ ] as suggested by the applicant.

   [X] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [ ] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
[redacted]/EP 00/01878

A. CLASSIFICATION OF SUBJECT MATT[redacted]
IPC 7 C12Q1/28

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MAIORINO M. ET AL.: "Testosterone mediates expression of the selenoprotein PHGPx by induction of spermatogenesis and not by direct transcriptional gene activation" FASEB J., vol. 12, 1998, pages 1359-1370, XP002141807 abstract page 1360, column 2, paragraph 4 page 1368, column 1, line 21-27 ---	1-4
X	WO 96 13225 A (BETH ISRAEL HOSPITAL ASSOCIATION) 9 May 1996 (1996-05-09) claims 12-19 ---	1-4

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 July 2000	25/07/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Pellegrini, P

1

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
[redacted]/EP 00/01878

C.(Continuation) DOCUMENTS CONSIDE[redacted]TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	ROVERI A. ET AL.: "Enzymatic and immunological measurements of soluble and membrane bound PHGPx" METHODS ENZYMOL., vol. 233, 1994, pages 202-212, XP000921475 cited in the application page 204, paragraph 5 -page 206, paragraph 2 ---	1-4
X	MAIORINO M. ET AL.: "Phospholipid hydroperoxide glutathione peroxidase" METHODS ENZYMOL., vol. 186, 1990, pages 448-457, XP000921458 page 452, paragraph 3 -page 455, paragraph 5 -----	1-4

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
EP 00/01878

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9613225 A	09-05-1996	US 5895749 A	20-04-1999
		AU 4018295 A	23-05-1996
		CA 2203828 A	09-05-1996
		EP 0789538 A	20-08-1997
		JP 11514204 T	07-12-1999
		US 5935800 A	10-08-1999

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 5-7

Claims 5-7 relate to an extremely large number of possible compounds (inhibitors of PHGPx), pharmaceutical compositions containing these compunds, and uses of these compounds and pharmaceutical compositions for revesibly blocking male fertility. However, support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is missing for the compounds, pharmaceutical compositions and uses claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. However, a search has been performed on the correlation between male fertility and PHGPx inhibition.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

International application No. PCT/EP 00/01878

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 5-7
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12Q 1/28 — A1

(11) International Publication Number: **WO 00/53800**

(43) International Publication Date: 14 September 2000 (14.09.00)

(21) International Application Number: PCT/EP00/01878

(22) International Filing Date: 6 March 2000 (06.03.00)

(30) Pri rity Data:
99103960.3 9 March 1999 (09.03.99) EP

(71)(72) Applicant and Inventor: FLOHÉ, Leopold [DE/DE]; Mascheroder Weg 1, D–38124 Braunschweig (DE).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: URSINI, Fulvio [IT/DE]; Mascheroder Weg 1, D–38124 Braunschweig (DE).

(74) Agents: BOETERS, Hans et al.; Boeters & Bauer, Bereiteranger 15, D–81541 München (DE).

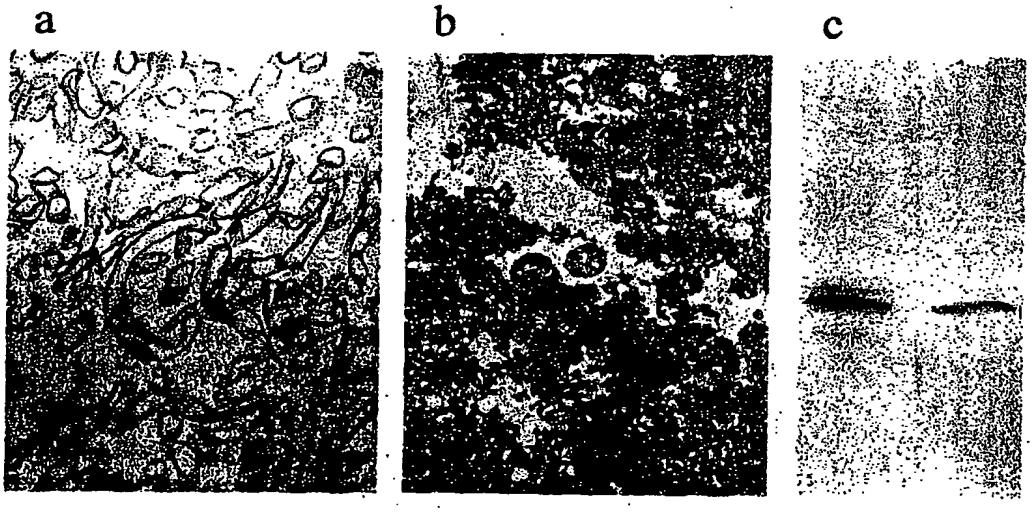
(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: METHOD TO SEARCH FOR MALE ANTIFERTILITY DRUGS BASED ON PHGPx ACTIVITY DETERMINATION



(57) Abstract

The invention relates to a method to search for male antifertility drugs based on activity determination of phospholipid hydroperoxide glutathione peroxidase (PHGPx) derived from human tissue or human cells or from related mammalian species.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

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Method to search for male antifertility drugs based on PHGPx activity determination

The invention relates to a method to search for male antifertility drugs based on activity determination of phospholipid hydroperoxide glutathione peroxidase (PHGPx) derived from human tissue or human cells or from related mammalian species.

Selenium is essential for male fertility. In mature mammalian spermatozoa it is largely restricted to the midpiece harbouring the helix of mitochondria embedded into a keratine-like selenium-enriched matrix called the mitochondrial capsule. Selenium deficiency is associated with impaired sperm motility, structural alterations of the midpiece up to breakages, and loss of *flagellum*. The predominant selenoprotein of the mammalian male reproductive system, phospholipid hydroperoxide glutathione peroxidase (PHGPx), was shown to be preferentially expressed in round spermatids but was hardly detectable in terms of messenger RNA or activity in spermatozoa. The inventors discovered that PHGPx persists in spermatozoa but as insoluble, enzymatically inactive material forming the mitochondrial capsule. PHGPx activity of this material can be

restored by high concentrations of thiols. PHGPx, thus, acts as a peroxidase in the proliferating germ epithelium to prevent oxidative damage. In the late stages of sperm maturation it is oxidatively cross-linked to become a structural element indispensible for sperm function. Based on this discovery the invention teaches to screen for specific inhibitors of PHGPx by activity measurements and to use such inhibitors for male fertility control.

The invention thus in accordance with claim 1 provides a method for screening for inhibitors of phospholipid hydroperoxide glutathione peroxidase (PHGPx) derived from human tissue or human cells comprising the steps of

a) determining the enzymatic activity of said PHGPx in the absence and presence, respectively, of at least one potential inhibitor,

b) selecting at least one inhibitor which specifically blocks PHGPx activity and subjecting said inhibitor(s) to a screening for pharmaceutical acceptance and

c) selecting a pharmaceutically acceptable inhibitor which, by specifically blocking PHGPx, reversibly suppresses male fertility.

In a further aspect the invention relates to a pharmaceutically acceptable inhibitor of PHGPx from human tissue obtainable by the inventive method and useful for male fertility control.

In yet another aspect the invention relates to a pharmaceutical composition comprising at least one such inhibitor of PHGPx from human tissue and at least one pharmaceutically acceptable carrier and/or diluent or no such carrier/diluent.

In still another aspect the invention relates to the use of such inhibitor of PHGPx or of such pharmaceutical composition comprising such inhibitor of PHGPx in a method for reversibly blocking male fertility.

Further advantageous and/or preferred embodiments of the invention are subject-matter of the subclaims.

Thus, the tissue or cells the PHGPx for screening is obtained from may be derived from life stock or any related mammalian species.

Further, the PHGPx may be produced by genetic engineering.

In addition, the potential inhibitors may have been tailored by computer designing and/or produced by a chemical process of production.

In the following the invention is disclosed in more detail with reference to examples and to drawings. However, the described specific forms or preferred embodiments are to be considered in all respects as illustrative and not restrictive, the scope of the invention being indicated by the appended claims rather than by the following description, and all changes which come within the meaning and range of equivalency of the claims are therefore intended to be embraced therein.

Regarding the cited literature a reference list with more detailed bibliographic information can be found at the end of this specification.

Routine preparations of rat sperm mitochondrial capsules (1) yielded a fraction which was insoluble in 1% SDS and 0.2 mM DTT and displayed expected vesicular appearance in electron microscopy (Fig. 1 a). The vesicles readily disintegrated upon exposure to 0.1 M mercaptoethanol (Fig. 1 b) and became fully soluble in 6 M guanidine-HCL. When the solubilized capsule material was subjected to gel electrophoresis essentially four bands in the 20 kDa region were detected (Fig. 1 c, left lane). Western blotting revealed that the most prominent one reacted with antibodies directed against PHGPx (Fig. 1 c, right lane) which is undetectable as active peroxidase in mature spermatozoa (Tab. 1). Also, N-terminal sequencing of the 21 kDa band representing about 46% of total protein content according to Coomassie stain revealed that it consisted of at least 95% pure PHGPx. Puzzled by this unexpected finding, we investigated the composition of the mitochondrial capsules in more detail by 2D-electrophoresis (Fig. 2 a) followed by microsequencing and/or MALDI-TOF for identification (Fig. 2 b). For this purpose the capsules were dissolved completely in a buffer designed for electrophoretic separation of membrane proteins (see Methods). The spot migrating with an apparent molecular weight of about 21 kDa and focussing at a pH near 8 (spot 3) proved to be PHGPx according to the masses of tryptic peptides detected by MALDI-TOF (Fig. 2 b). By the same technique, also the slightly more acidic charge isomer (spot 4), the more basic ones (spots 1, 2 and 5) as well as the spots 6 and 7 exhibiting a smaller apparent molecular mass were shown to contain PHGPx (Fig. 2 c). The predicted N-terminal (pos. 3-12) and C-terminal peptides (pos. 165-170), the fragment corresponding to positions 100-105 and those expected from the basic sequence part 119-151 were too small to

be reliably identified. Interestingly, the fragment corresponding to positions 34-48 comprising the active site selenocysteine was not detected either. With these exceptions, however, the MALDI-TOF spectra unequivocally complied with the PHGPx sequence and thus proved the presence of PHGPx in spots 1-7. On a thicker 2D-gel developed with a non-linear gradient from pH 3-10 also five distinct spots were detected in the 20 kDa region. In this experiments the presence of PHGPx was verified by microsequencing of major tryptic peptides (not shown). Again the spots representing PHGPx were the most prominent ones present in the gel.

The spots 1-6 of Fig. 2 a proved to be essentially homogeneous. As is exemplified in Fig. 2 b, the fragments yielding MALDI-TOF signals of significant intensities could be attributed to PHGPx. Only in the minor spot 7 a trace of impurity was detected, which was tentatively identified as a subunit of the T cell receptor variable region (acc. no. 228109). Based on integrated stain intensities of the individual spots those representing PHGPx amounted to about 50% of the capsule material. Most of the minor components (see Fig. 2 a) are not likely constituents of the capsule, which is believed to be built up by apposition of extramitochondrial proteins onto the outer mitochondrial membrane. In other gels further proteins like the mitochondrial glutathione S-transferase subunit Yb-2 (acc. no. 121719) and an endothelin converting enzyme (acc. no. 1706564) could be identified by MALDI-TOF or micro-sequencing (not shown). Spots 8 and 9 were identified as the "outer dense fiber protein", a cystine-rich structural sperm protein, which is associated with the helix of mitochondria in the sperm midpiece but also extends into the *flagellum* (7). In view of the nature of the additional proteins

detected, the PHGPx content of the actual mitochondrial capsule should substantially exceed the 50% observed by gel scanning.

Despite intense search, we could not detect any trace of the "sperm mitochondria-associated cysteine-rich protein ("SMCP") (7) in our capsule preparation. This cysteine- and proline-rich protein had for long been considered the selenoprotein accounting for the selenium content of the mitochondrial capsule in sperm (1,8,9). Cloning of the rat SMCP gene, however, revealed that it did not contain any in-frame TGA codon enabling selenocysteine incorporation (10). In mice, the three in-frame TGA codons proved to be upstream of the translation start (7). In developing mouse sperm SMCP stayed cytosolic up to states in which the mitochondrial capsule was already formed and only became superficially associated with the outer mitochondrial membranes of late spermatids and epididymal spermatozoa (7). SMCP thus is not necessarily an integral part of the mitochondrial capsule nor it is a selenoprotein. Instead, the "mitochondrial capsule selenoprotein (MCS)", as SMCP was originally referred to (1,7-10), is indeed PHGPx.

The chemical modifications of PHGPx leading to distinct differences in charge and apparent MW could not be reliably elucidated. Sequencing revealed an identical N-terminus of the size isomers starting with ASRDDWRCAR, i.e. a sequence either corresponging to the originally proposed translation start (11) after cleavage of the first two residues or derived from a possible pre-PHGPx (12) after processing of a mitochondrial leader peptide (13). Tryptic fragments extending towards the C-terminus up to position 164 were consistently observed al-

so with the faster migrating specimen (Fig. 2 c) which leaves little room to explain an apparent MW difference of 1 to 1.5 kDa. As to the charge isomers, it may be recalled that a potential phophorylation had been inferred from early attempts to sequence pig heart PHGPx (14). The assignment of masses to possibly phosphorylated tryptic peptides, however, remained equivocal. Certainly, more trivial events such as deaminations of Gln and Asn residues, C-terminal degradation, oxidation of the active site selenium, or its elimination might have contributed to the charge heterogeneity.

PHGPx as the major component of the sperm mitochondrial capsule had so far escaped attention, since as such it is enzymatically inactive, as it generally is in mature spermatozoa prepared from the tail of the epididymis (Tab. 1). It is neither reactivated by glutathione in the low millimolar range as used under conventional test conditions. High concentrations of thiols (0.1 M 2-mercaptoethanol or dithiothreitol), which in the presence of guanidine fully dissolve the capsule, regenerate a significant PHGPx activity, as measured after elimination of denaturating and reducing agents (Tab. 1). In fact, the specific activities thus obtained from mitochondrial capsules exceed, by a factor of 20, the highest values ever measured, i.e. in spermatogenic cells. Nevertheless, this extreme PHGPx activity is still low compared to its content in PHGPx protein. Based on the specific activity of pure PHGPx, the reactivated enzyme would be equivalent to less than 3% of the capsule protein, whereas the 2D-electrophoresis suggests a PHGPx protein content of at least 50%. It is worth noting that the same reductive procedure does not increase the specific activity of PHGPx in spermatogenic cells from testicular tubules (Tab. 1). The switch of

PHGPx from a soluble active enzyme to an enzymatically inactive structural protein thus occurs during final differentiation of spermatozoa.

The alternate roles of PHGPx, being either a glutathione-dependent hydroperoxide reductase or a structural protein, are not necessarily unrelated. One of the features common to all glutathione peroxidases is a selenocysteine residue which together with a tryptophan and a glutamine residue forms a catalytic triad (15,16). Therein the selenol group of the selenocysteine residue is dissociated and highly activated by hydrogen bonding to reduce hydroperoxides with high rate constants. The reaction product, a selenenic acid derivative, R-SeOH, will readily react with thiols, e.g. GSH, to form an intermediate with a selenadisulfide bridge between enzyme and substrate, R-Se-S-G, from which the ground state enzyme can be regenerated by a second GSH. PHGPx is unique among the glutathione peroxidases in several respects: i) It usually is monomeric having its active site freely accessible at the surface; this facilitates interaction with bulky substrates. ii) Arginine residues surrounding the active site and specifically binding glutathione in most types of glutathione peroxidases are lacking in PHGPx (16); correspondingly, its specificity for the reducing substrate is less pronounced (16). It therefore can be envisaged that oxidized PHGPx may form diselenide or selenadisulfide bridges with exposed SeH or SH groups of proteins (16) including PHGPx itself, and this process, possibly followed by SH/SS, SH/SeS, or SH/SeSe exchange reactions, will create cross-linked protein aggregates. This ability of PHGPx might become particularly important if cells are exposed to hydroperoxides at extremely low concentration of glutathione, as is documented for late

states of spermatogenesis (17-20). Fig. 3 is to mimick the oxidative events occurring during sperm maturation. Short term exposure of soluble proteins derived from spermatogenic cells to moderate H_2O_2 concentrations in the absence of GSH yields a variety of PHGPx-containing high molecular weight aggregates. Undoubtedly, therefore, PHGPx, by means of its intrinsic enzymatic potential, can catalyse oxidative protein aggregation using protein thiols as alternate substrates. During sperm maturation, PHGPx thereby transforms itself into an enzymatically inactivated structural protein. This view, however, is not to imply that PHGPx could not depend on additional proteins when building up the highly organized architecture of the spermatozoal midpiece.

Our findings require a fundamental reconsideration of the role of selenium in male fertilty. The intriguing predominance of the selenoprotein PHGPx in the male reproductive system has so far been believed to reflect the necessity to shield germ line cells from oxidative damage by hydroperoxides and reactive oxygen species derived therefrom (11,17,21,22). This concept still merits attention with regard to the mutagenic potential of hydroperoxides and probably holds true for the early phases of spermatogenesis where PHGPx is still present as an active peroxidase (6,21). At this stage related activities reported for PHGPx or other glutathione peroxidases, e.g. silencing lipoxygenases (23), dampening the activation of NFκB (24) or inhibiting apoptosis (25), may also be relevant. In later stages of spermatogenesis characterized by a shift of the redox status resulting in loss of GSH (18-20,26), the ability of PHGPx to use protein thiols as alternate substrates opens up new perspectives of redox regulation which remain to be explored. In the mature spermatozoon PHGPx

has experienced a pronounced metamorphosis now being a major constituent of the keratinuous material embedding the mitochondrial helix. It appears revealing that precisely this architectural pecularity in the midpiece of spermatozoa shows gross structural alterations in selenium deficiency. We therefore assume that the mechanical instability of the midpiece observed in selenium deficiency is a consequence of an impaired PHGPx biosynthesis. This view implies that it is not the antioxidant capacity of PHGPx which is crucial for male fertility but its ability to utilize hydroperoxides to build an indispensable structural element of the spermatozoon.

Any shortage of PHGPx during sperm maturation, be it due to selenium deficiency, other reasons of inhibited biosynthesis or inhibition of activity should therefore result in disturbed sperm midpiece architecture and, in consequence, loss of fertilization potential of sperm. This conclusion was further corroborated by determination of reactivated PHGPx in sperm of individuals with documented fertility problems. The latter were divided into three groups: depending on whether i) intrauterine sperm injection (iui) or ii) conventional in-vitro-fertilisation (ivt-et) was still successful or iii) intracytoplasmatic sperm injection was required (icsi). As shown in Fig 4, the PHGPx values differed markedly between these groups. While the iui group displayed values close to normal, PHGPx in the icsi group was almost absent, the ivf-et group ranking in between. The reasons of the diverse PHGPx content being unknown, the data reveal that markedly reduced PHGPx content in sperm is incompatible with normal male fertility. Similarly, there is a strong correlation between "typical" sperm appearance (Fig 5) and "fast" moving sperm with PHGPx content (Fig 6). This correlation, however, shows mar-

ked scattering of data indicating that PHGPx content of sperm is not the only reason of abnormal shape and motillity of sperm. It should also be pointed out that the sperm samples were taken from individuals without any obvious disease suggesting that extremely reduced PHGPx levels are well tolerated.

Taken together, the observations that PHGPx builds up an essential structure of sperm, that PHGPx content of sperm correlates with the fertilization potential and that severe PHGPx deficiency does not cause any systemic health problems lead to the inventive concept to use PHGPx as a molecular target enzyme for screening of specific inhibitors which should reversibly block the fertilization potential of sperm. To this end it appears irrelevant whether reactivated PHGPx of sperm or any other tissue is used, since the characteristics of the enzyme do not differ between tissues. With respect to the high degree of PHGPx sequence conservation also heterologous PHGPx may successfully be used for a preliminary screen of potential inhibitors of the human enzyme. The search for such inhibitors can be performed with any of the known assays for activity determination of glutathione peroxidases (29) or modifications thereof, which may be adapted to high-throughput screening procedures (30, 31).

Methods

Preparation of rat spermatozoa, tubular cells and mitochondrial capsule

Spermatozoa of four month old Wistar rats (about 300 grams of body weight) were collected by squeezing *cauda epididymis* and *vas deferens* in phosphate buffer saline (PBS) and by centrifugating at 600 x g for 10 minutes. Cell and sperm pellets were layered on a discontinous 45%, 70% and 95% Percoll gradient and centrifugated at 300 x g for 20 min. Spermatogenic cells stacked on top of the gradient, while spermatozoa separated into the 70% Percoll layer. Cells from seminiferous epithelium were prepared as follows (26): testes were deprived of *albuginea*, seminiferous tubules were cut into small pieces in PBS containing 0.250 mg/ml collagenase, and incubated twice 25 °C for 15 min. Cells then were filtered through a stainless steel screen (140 µm pore), washed in PBS and centrifugated at 300 x g for 10 min. Sperm mitochondrial capsule was prepared according to Calvin et al.(1): sperms were resuspended in 0.05 M Tris - HCl pH 8.0 at the concentration of 10^6 cells/ml and treated with trypsin (0.2 mg/ml) for 10 minutes. After stopping the protease action with trypsin inhibitor (0.5 mg/ml) and SDS (10 mg/ml) sperms were centrifugated at 1,500 x g for 10 minutes. Pellets were resuspended in 0.05 M Tris - HCl, pH 8.5 containing 1% sodium dodecyl sulphate (SDS), and 0.2 mM DTT and kept under continuous stirring for 30 minutes. Following centrifugation at 4,500 x g for 15 min, the resulting supernatant was layered on a 1.6 M sucrose cushion. After centrifugation for 20 min at 18,000 x g in a swinging rotor, sperm capsules were collected as a band at the top of the sucrose cushion, washed in Tris - HCl, pH 8.0 and spun at 140,000 x g.

1D-electrophoresis and Western blotting

Electrophoresis was performed according to Laemmli under either reducing (+ 2- mercaptoethanol) or non-reducing conditions. Proteins were blotted onto nitrocellulose, probed with an antigen-purified rabbit antibody raised against pig heart PHGPx and detected by biotinylated anti rabbit IgG and streptoavidin alkaline phosphatase complex.

2D-electrophoresis

100µg of the mitochondrial capsule material was dissolved in 400 µl of a solution containing of 7 M urea, 2 M thiourea, 4% CHAPS, 40 mM DTT, 20 mM Tris base and 0.5% IPG buffer (Pharmacia) and focused in an IPG-phor (Pharmacia) at 20°C by stepwise increasing voltage up to 5000 V but not exceeding a current of 30 µA per IPG strip. The pH gradient was non-linear from 3-10 or linear from 3-10 or 6-11. The focussed IPG strips were then equilibrated for SDS electrophoresis (10 min each) with a solution containing 60 mM DTT in 6 M urea, 30% glycerol, 0.05 M Tris-HCl buffer pH 8.8 and in the same buffer where DTT was substituted by 250 mM iodoacetamide. After SDS-electrophoresis (12% polyacrylamide) the gels were stained with Coomassie.

Protein identification

Coomassie-stained spots were cut out from the gels, neutralized with $(NH_4)HCO_3$, destained with 400 µl 50% acetonitrile/10 mM $(NH_4)HCO_3$ and dried in a Speed Vac Concentrator. Protein digestion was done overnight using 2 ng/µl sequencing grade trypsin (Promega) in 50 mM $(NH_4)HCO_3$ (Boehringer, Mannheim). The resulting peptides were extracted twice with 60% acetonitrile / 40% H_2O / 0.1% TFA. Extracts were combined and lyo-

philized in the Speed Vac Concentrator. Peptide digests were desalted on small RP18-columns, eluted with saturated α-hydroxy-4-cyano-cinnamic acid and loaded directly onto the MALDI target (27). Reflectron MALDI mass spectra were recorded on a Reflex™ MALDI/TOF-mass spectrometer (Bruker-Franzen-Analytik, Bremen). The ions were excellerated at 20 kV and reflected at 21.3 kV. Spectra were externally calibrated using the monoisotopic MH^+ ion from two peptide standards. 100-200 laser shots were summed up for a single mass spectrum. Mass identifcation was performed with MS-Fit (http://falcon.ludwig.ucl.ac.uk/ucsfhtml/msfit.htm).

Alternatively, protein spots from 1.5 mm 2D-gels were digested with modified trypsin (Promega, sequencing grade) in 25 mM $(NH_4)HCO_3$ overnight at 37°C. The digests were extracted twice and dried as before and reconstituted in 10µl water. Peptides were separated on a reversed-phase capillary column (0.5 mm x 150 mm) with a gradient of acetonitrile in 0.1% formic acid / 4 mM ammonium acetate at a flow rate of 5µl/min and collected manually. Aliquots of 5 µl were spotted onto Biobrene-treated glass fiber filters and sequenced on an Applied Biosystems 494A sequencer with standard pulsed-liquid cycles. Before N-terminal sequencing, proteins were blotted from polyacrylamide gels onto PVDF membranes for 16 h at pH 8.3 (25 mM Tris-HCl, 192 mM glycine) and 100 mA (30 V).

When applicable, PHGPx was also identified by activity measurement according to (28) using the specific substrate phosphatidylcholine hydroperoxide.

Figure 1 shows the presence of PHGPx in the mitochondrial capsule of spermatozoa.

a, Mitochondrial capsule prepared by trypsination and centrifugation according to (1) at 80,000 fold magnification. **b**, The same preparation as shown in **a**, but after exposure to 0.1 M 2-mercaptoethanol for 15 min at 4°C. Contamination of the capsule material by mitochondria is evident from the presence of mitochondrial ghosts. **c**, SDS gel electrophoresis of proteins extracted from capsule material (see Methods) by treatment with 0.1 M 2-mercaptoethanol, 0.1 M Tris-HCl, pH 7.5, and 8 M guanidine HCl. Left lane is stained with Coomassie, right lane demonstrates presence of PHGPx by Western blotting.

Figure 2 shows the analysis of the composition of the mitochondrial capsule of spermatozoa

a, 2D-electrophoresis of purified dissolved capsule material. Proteins were focused in a linear pH-gradient from 3 to 10 (horizontal direction), then reduced, amidocarboxymethylated, subjected to SDS-electrophoresis, and stained with Coomassie. MALDI-TOF analysis of the visible spots identified the following proteins (SwissProt data base): spot 1-7 PHGPx (MW 19 443; pI 8.27; acc. no. 544434); spots 8 and 9, outer dense fiber protein (MW 27351; pI 8.36; acc. no. P21769); spots 10 and 11, voltage-dependent anion channel-like protein (MW 31720; pI 7.44; acc. no. 540011); spot 12, "stress-activated protein kinase" (MW 48107; pI 5.65; acc. no. 493207); spot 13, glycerol-3-phosphate dehydrogenase (MW 76479; pI 5.86; acc. no. P35571).

b, MALDI-TOF spectrum (overview) of tryptic peptides obtained from PHGPx as found in spot 3. Abscissa, mass/charge ratio

of the peptide fragments; ordinate, arbitrary units of intensity; numbers at mass signals, identified peptides in the PHGPx sequence (see insert for position numbers); T, trypsin-derived fragments.

c, Compilation of tryptic PHGPx fragments identified in spots 1-7 by MALDI-TOF. Vertical lines designate potential tryptic cleavage sites. Dark blocks, identified typical cleavage products; shadowed blocks, masses resulting from incomplete cleavage or equivocally assignable to different fragments (e.g. 3-9 and 63-69).

Figure 3 shows the formation of PHGPx-containing aggregates from spermatogenetic cells by H_2O_2 in the absence of GSH. Spermatogenic cells were homogenised in 0.1 M Tris-HCl, 6 M guanidine-HCl, 0.5 µg/ml pepstatin A, 0.7 µg/ml leupeptin and 5mM 2-mercaptoethanol at pH 7.5 and 4°C. After centrifugation at 105,000 x g for 30 min, excess reagents were removed by gel permeation using NAP 5 columns equilibrated with 10mM Tris-HCl, 0.15 M NaCl, 1mM EDTA and 0.1% Triton X-100, pH 7.5. Immediately (t 0) and 15 min after (t 15) the addition of 75 µM H_2O_2 aliquots of the mixture (0.05 mg of protein) were withdrawn and subjected to electrophoresis under **(a)** reducing and **(b)** non reducing conditions. After blotting on nitrocellulose, PHGPx was detected by specific antibodies.

Figure 4 shows the PHGPx specific activity in extracts (0.1% Triton X-100 and 0.1 M 2-mercaptoethanol of human sperm. Correlation between this parameter and therapeutic appproach in cases of couple infertility.

Figure 5 shows the relationship between PHGPx specific activity and number of "typical" sperms per milliliter of semen. "Typical" is a morphological parameter of sperm evaluation.

Figure 6 shows the relationship between PHGPx specific activity and number of "fast" sperms per milliliter of semen. "Fast" is a parameter of sperm mobility.

Table 1 shows PHGPx activity in spermatogenic cells, spermatozoa and sperm capsule. Effect of thiols.

Preparation	mU/mg protein [a, b]
Cells from seminiferous tubules	
5 mM 2-mercaptoethanol [c]	250 ± 10
100 mM 2-mercaptoethanol [c]	260 ± 10
Spermatozoa from tail of epididymis	
5 mM 2-mercaptoethanol [c]	undetectable
100 mM 2-mercaptoethanol [c]	3,140 ± 200
Mitochondrial capsule	
5 mM 2-mercaptoethanol [c]	undetectable
100 mM 2-mercaptoethanol [c]	5,600 ± 290

[a] One enzyme mU catalyzes the reduction of one nanomole of phosphatidylcholine hydroperoxide per minute at 37 °C in the presence of 3 mM GSH.

[b] Data are the mean and S. D. of five independent measurements.

[c] Solubilisation / reduction was carried out in 0.1 M Tris-HCl, 6 M guanidine-HCl, 0.5 µg/ ml pepstatin A, 0.7 µg/ml

leupeptin and 2-mercaptoethanol as indicated at pH 7.5 and 4 °C for 10 min Low molecular weight compounds were removed before activity determination by a NAP 5 cartridge.

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Claims

1. Method for screening for inhibitors of phospholipid hydroperoxide glutathione peroxidase (PHGPx) derived from human tissue or human cells comprising the steps of
   a) determining the enzymatic activity of said PHGPx in the absence and presence, respectively, of at least one potential inhibitor,
   b) selecting at least one inhibitor which specifically blocks PHGPx activity and subjecting said inhibitor(s) to a screening for pharmaceutical acceptance and
   c) selecting a pharmaceutically acceptable inhibitor which, by specifically blocking PHGPx, reversibly suppresses male fertility.

2. Method of claim 1, wherein the tissue or cells are from life stock or any related mammalian species.

3. Method of claim 1, wherein PHGPx is produced by genetic engineering.

4. Method of claim 1, 2 or 3, wherein the potential inhibitors have been tailored by computer designing and/or produced by a chemical process of production.

5. A pharmaceutically acceptable inhibitor of PHGPx from human tissue obtainable by the method according to claim 1, 2, 3 or 4 and useful for male fertility control.

6. Pharmaceutical composition comprising at least one inhibitor of PHGPx from human tissue according to claim 5

and at least one pharmaceutically acceptable carrier and/or diluent or no such carrier/diluent.

7. Use of an inhibitor of PHGPx according to claim 5 or of a pharmaceutical composition comprising said inhibitor of PHGPx according to claim 6 in a method for reversibly blocking male fertility.

FIG.1

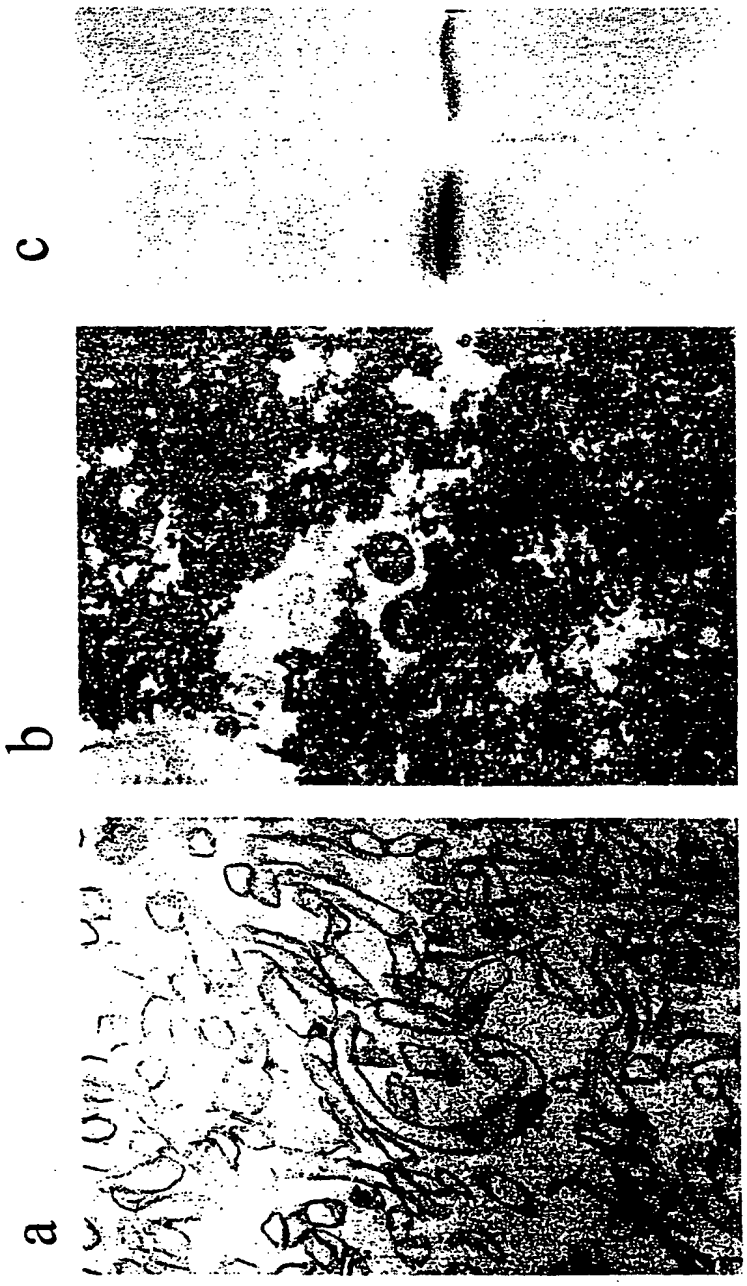
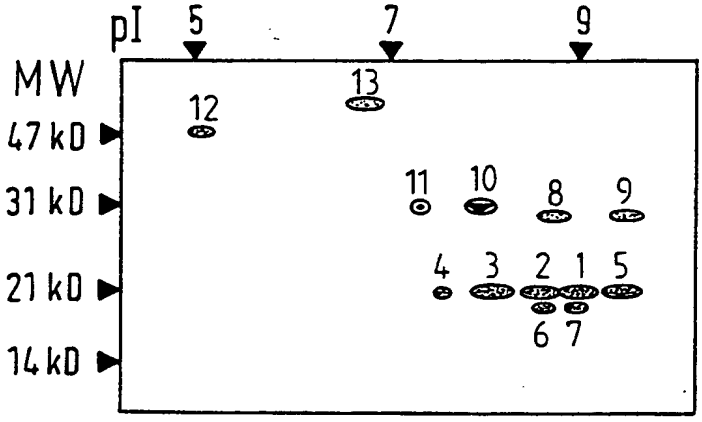
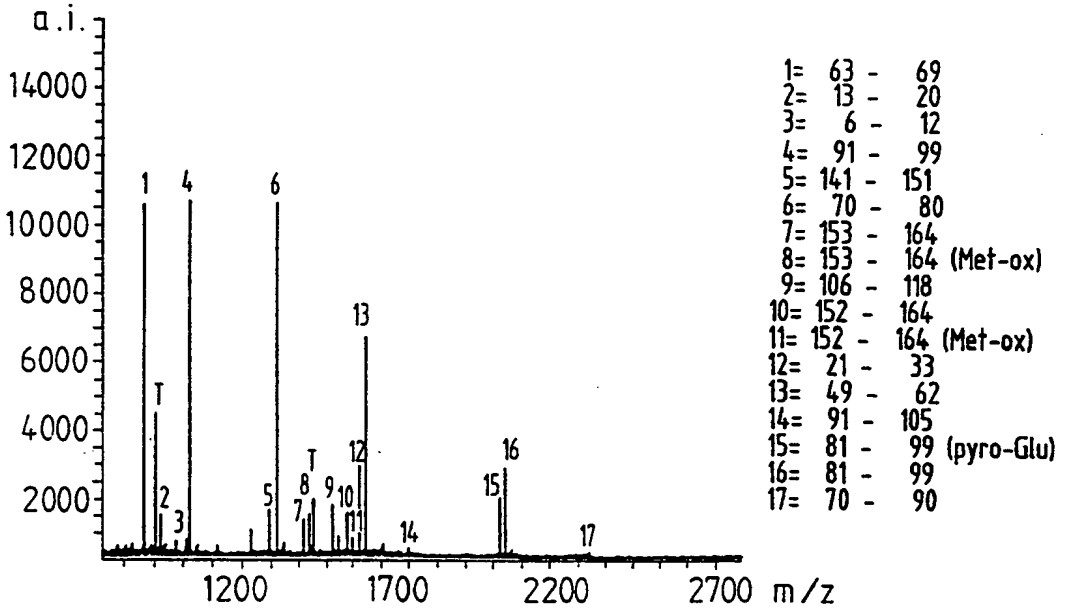


Fig. 2

a)


pI
5
7
9
MW
47 kD
31 kD
21 kD
14 kD
12
13
11
10
8
9
4
3
2
1
5
6
7

b)


a.i.
14000
12000
10000
8000
6000
4000
2000
1200
1700
2200
2700
m/z
1= 63 - 69
2= 13 - 20
3= 6 - 12
4= 91 - 99
5= 141 - 151
6= 70 - 80
7= 153 - 164
8= 153 - 164 (Met-ox)
9= 106 - 118
10= 152 - 164
11= 152 - 164 (Met-ox)
12= 21 - 33
13= 49 - 62
14= 91 - 105
15= 81 - 99 (pyro-Glu)
16= 81 - 99
17= 70 - 90

c)

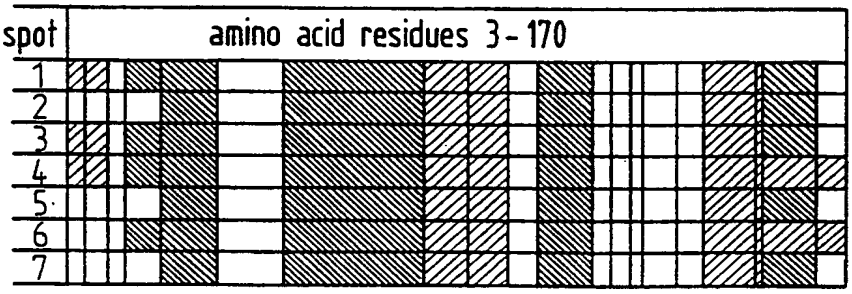

spot
amino acid residues 3 - 170
1
2
3
4
5
6
7

Fig. 3

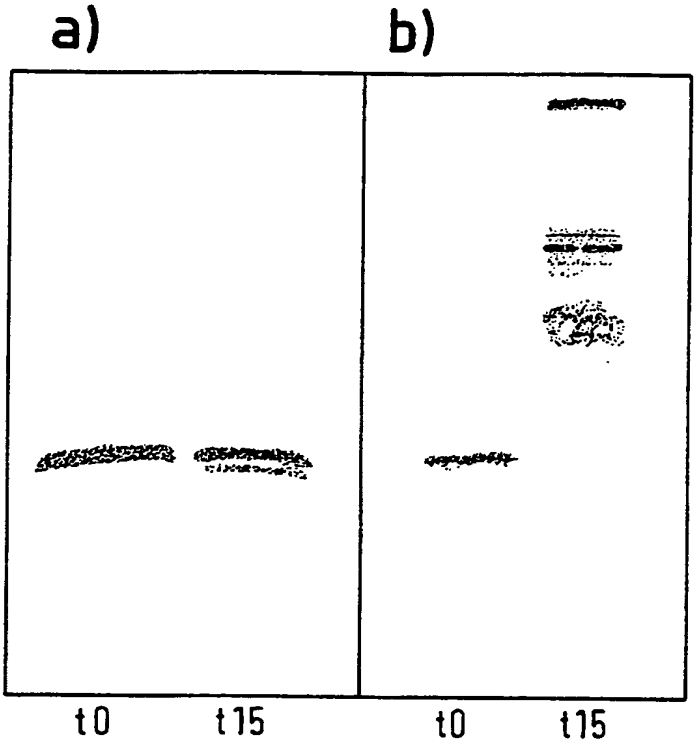

a)
b)
t0
t15
t0
t15

Fig. 4

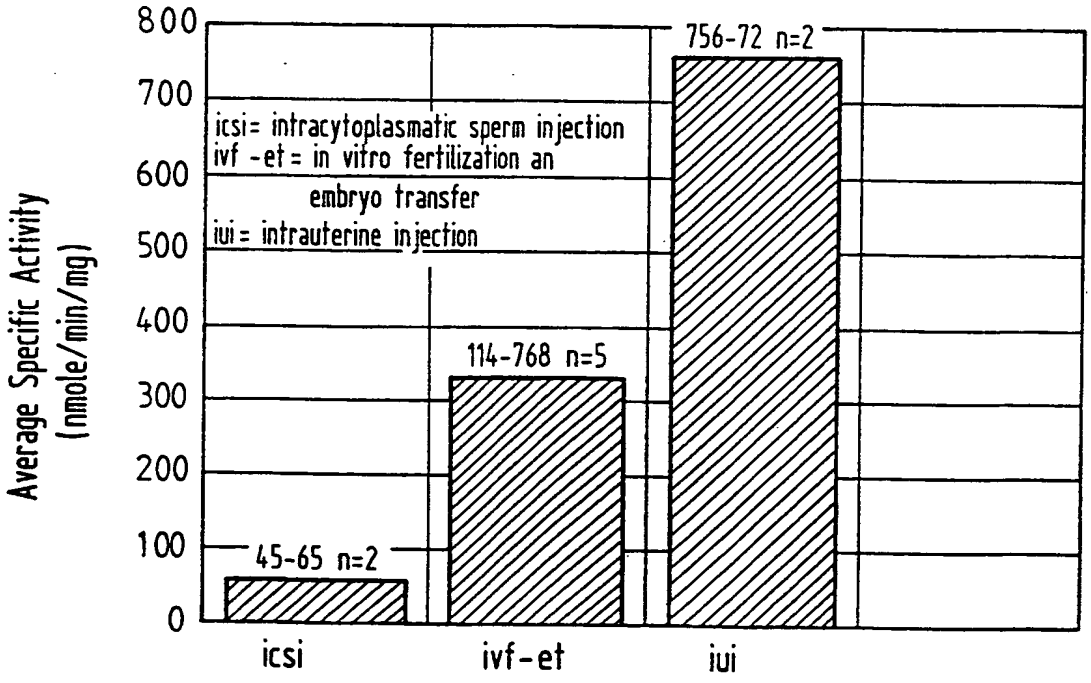

800
700
600
500
400
300
200
100
0
Average Specific Activity (nmole/min/mg)
icsi= intracytoplasmatic sperm injection
ivf -et = in vitro fertilization an embryo transfer
iui = intrauterine injection
45-65 n=2
114-768 n=5
756-72 n=2
icsi
ivf-et
iui

Fig. 5

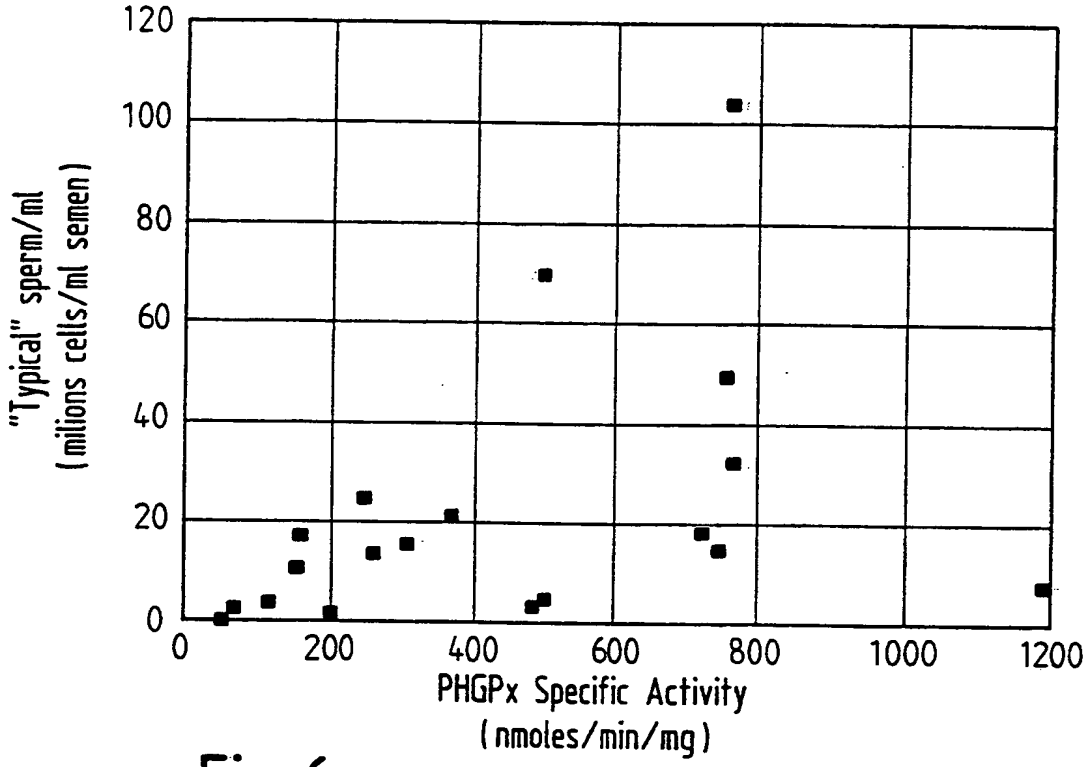
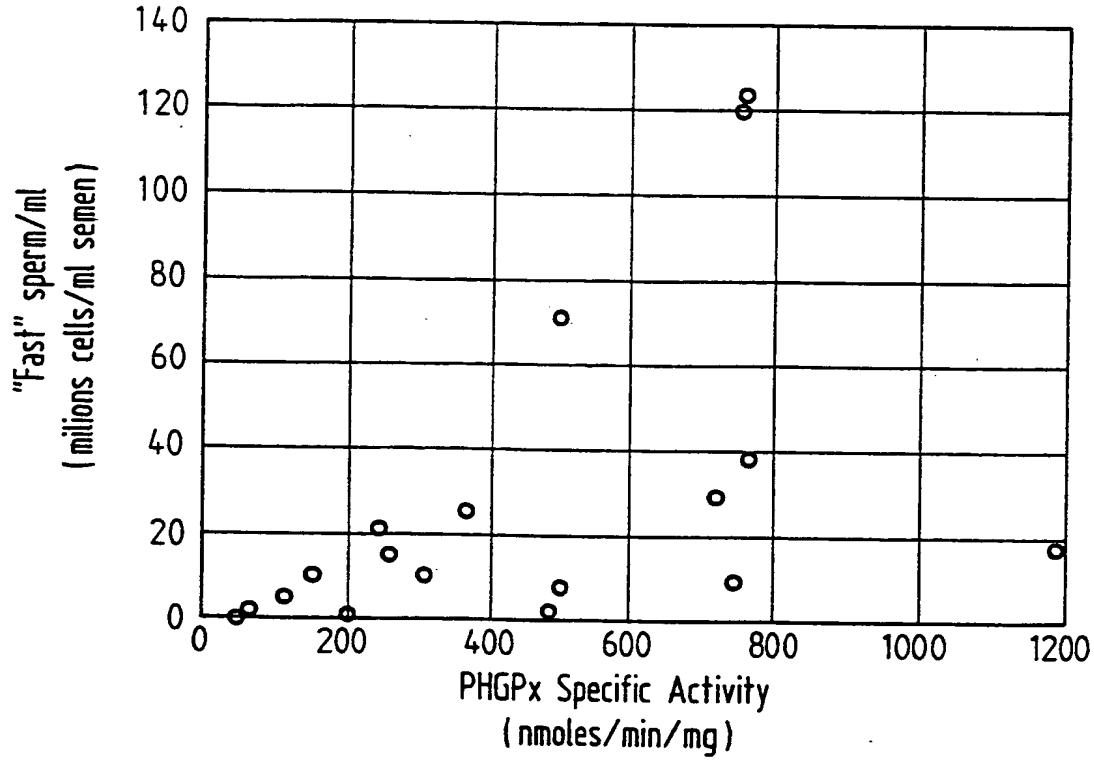

"Typical" sperm/ml
(milions cells/ml semen)
120
100
80
60
40
20
0
0
200
400
600
800
1000
1200
PHGPx Specific Activity
(nmoles/min/mg)

Fig. 6


"Fast" sperm/ml
(milions cells/ml semen)
140
120
100
80
60
40
20
0
0
200
400
600
800
1000
1200
PHGPx Specific Activity
(nmoles/min/mg)